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A novel method for analysis of the periodicity of chondrogenic patterns in limb bud cell culture: correlation of in vitro pattern formation with theoretical models

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Key Words: Pattern formation, Micromass culture, Image analysis, Computer simulation

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Abstract

To experimentally examine whether the pattern changes predicted by theoretical models of pattern formation actually occur in a limb bud cell culture system, we developed a practical method to automatically measure the periodicity of chondrogenic patterns in vitro. The method utilizes binary image processing to quantify the total number of peak and valley pixels in a pattern to obtain the average interval between stripes in the chondrogenic pattern, and we named it the peak length method. The reliability of the peak length method was examined by using computer simulation results. The peak length method enabled to quantitatively obtain the average interval between chondrogenic islands, and the values obtained by this method were closely correlated with the average intervals obtained by manual measurement and two-diomensional Fourier transform. The average intervals obtained by the peak length method were shown to be stable over a wide range of pattern variations which are frequently observed in actual experiments. By applying the peak length method to actual experimental data, we compared the validity of two theoretical models of pattern formation (cell sorting model and reaction-diffusion model) and it was concluded that this method could be a useful tool to quantitatively analyze chondrogenic patterns in limb micromass culture and to relate theoretical predictions and experimental results of pattern formation.

Introduction

The pattern formation in vertebrate limb development is a major target of research in developmental biology and has been extensively studied both experimentally and theoretically. So far, experimental studies have focused mainly on the *polarity* in the axes of the limb. Recent studies employing molecular biological techniques have revealed the roles of molecules responsible for establishing the polarity in the limb axes. For example, the proximo-distal growth of the limb appears to be regulated by the fibroblast growth factor (FGF) family of proteins. The anterior-posterior axis seems to be regulated by the Sonic hedgehog (Shh) protein, and the dorso-ventral axis, at least in part, by Wnt-7a (Gilbert, 1997).

On the other hand, theoretical studies have focused mainly on the *periodic* aspect of the chondrogenic patterns in the developing limb bud and some theoretical models have been proposed to explain the mechanisms of pattern formation in limb chondrogenesis. For example, Newman and Frisch (1979) suggested that the reaction-diffusion model which was originally proposed by Turing (1952) could be applied to limb chondrogenesis. Oster et al. (1983) proposed a model which included the mechanical aspect of extracellular matrices (ECMs) to explain some properties of pattern formation in vivo. Manoussaki et al. (1996) applied the model to vasculogenesis in vitro and theoretically explained the experimental observation that pattern changes occur when ECM thickness was changed (Vernon et al. 1992). In spite of their efforts to theoretically explain the mechanisms of pattern formation, these models have seldom been tested in living animals because the vertebrate limb is an extremely complex and asymmetric organ and its morphogenesis involves various complicated developmental phenomena.

Micromass culture of limb bud cells is an in vitro model system that replicates in vivo differentiation of chondrogenic cells. The roles of various molecules involved in the differentiation of limb chondrocytes have been elucidated by using this culture system. For example, transforming growth factors (TGFs), cadherins, and ECMs act positively for chondrocyte differentiation (Schofield and Wolpert, 1990; Chimal Monroy and Diaz de Leon, 1997; Kulyk et al., 1989; Oberlender and Tuan, 1994; Seghatoleslami and Kosher, 1996) while Wnt-7a acts negatively (Rudnicki and Brown, 1997). It has also been shown that retinoic acid (RA) has biphasic effects on the differentiation of chondrogenic cells. At low concentrations RA promotes their differentiation, but it inhibits differentiation at high concentrations (Ide and Aono, 1988).

Micromass culture may be a potentially useful tool to experimentally validate theoretical models because this system is said to retain the *periodic* nature of chondrogenic patterns in vivo (Newman, 1996). In this culture system, some cells differentiate to become chondrocytes while others remain fibroblastic, and a whorl-like pattern is formed by chondrocytes (Fig. 1A). This in vitro system has usually been used as an easy assay for chondrogenic differentiation and for toxicological screening, and the potential of this system to study pattern formation has been largely ignored. However, some investigators studied the mechanisms of pattern formation in this culture system. For example, Downie and Newman (1995) hypothesized that the chondrogenic pattern is generated by the reaction-diffusion mechanism and experimentally examined the roles of fibronectin and TGFß as possible "activator" molecules. Mochizuki et al. (1996) created a

computer simulation based on the cell sorting mechanism and proposed two statistics based on the model to estimate the cell-cell affinity from the observed pattern.

To experimentally validate these theoretical models, some methods are needed to analyze the *periodicity* in the chondrogenic pattern because it is one of the most important properties explained by theoretical models. In the present study, we defined a simple parameter, named "the average interval", to represent the periodicity in the chondrogenic pattern, and developed a practical method to automatically measure the average interval in the patterns observed in the micromass culture of limb bud cells. The method utilizes binary image processing to quantify the total length of peaks and valleys in chondrogenic patterns to estimate the average interval between stripes formed by chondrocytes. We also examined the validity of this method by comparing the obtained values with that obtained by Fourier transform method, which is mathematically well-established (Russ, 1992) but not appropriate for actual experimental images which contains a distortion, in various idealized simulation images, and showed that this method can be applied to actual experimental data to compare two models of pattern formation, the cell sorting and reaction-diffusion models. The method was implemented in the public domain NIH Image program (developed at U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image), an image processing software for the Macintosh and PC, which can be easily introduced for routine analysis. We propose that this method can be a useful tool to correlate experimental and theoretical studies of periodic pattern formation.

Materials and Methods

Limb micromass culture

Limb micromass culture was carried out by the method described previously (Ownes and Solursh, 1981). ICR mouse embryos were used for preparation of all mouse cell suspensions. Pregnant day-10.5 dams (noon of the plug day = day 0.5) were killed by cervical dislocation and their uteri were removed and placed in calcium-magnesium free Tyrode solution. Then forelimb buds of live embryos were dissected with microscissors, washed several times with Tyrode solution and incubated with 1% trypsin for 20 min., at 37 °C. After limb buds were washed with Tyrode solution for 2-3 times, cells were dissociated by pipetting and filtered with a nylon mesh filter of the pore size of 10 μm. After that, the cell density was measured with a hemocytometer, and the suspension was cytocentrifuged to make a final concentration of 1.0×10^7 cells/ml.

Ten microliter drops containing 1.0×10^5 cells were placed on to the 35-mm Falcon tissue culture dish. The dish had been coated with collagen I or fibronectin and its bottom surface was incised with a trepan beforehand to make drops of the same size (4 mm in diameter). The cells were allowed to attach to the dish for approximately 2 Hrs in humidified atmosphere at 37 °C. After that, each culture was flooded with 2 ml of the medium which contains vehicle control (PBS), 0.25mM EDTA or 500 μM Arg-Gly-Asp (RGD) peptide. After incubation for 5 days, the cells on the dish were fixed in 4% PFA overnight and stained with pH 1.0 Alcian blue.

Definition of the average interval

To assess the periodicity in a given chondrogenic pattern, we first manually measured the distances between the nearest neighboring peaks (center lines) of the chondrogenic areas (Fig. 1B) or between the centers of spots if the pattern contains a spot-like part, and defined their mean value as the average interval. The interval did not depend on the width of the chondrogenic areas. This value was stable when a sufficient number of intervals were measured. We usually measured 50 different sets of points in each sample for statistical comparison. We used this method to asses the periodicity of the pattern in a previous report (Miura and Shiota, 1999)

Although this method is intuitively easy to understand, it may imply two substantial problems. First, as seen in Fig. 1B, there is arbitrariness in choosing the distances between neighbouring centers. The selection might be influenced by the observer's subjectivity. Second, the measuring process is time-consuming and it is hard to analyze a large number of images with this method.

Fourier transform

Because of the problems mentioned above, we used the two-dimensional Fourier transform to validate the definition of the average interval. Fourier transform is a well established standard mathematical method to analyze periodicity (Russ, 1992), and its basic idea is to detect the highest amplitude in the spatial frequency domain, which is likely to represent the dominant periodicity in chondrogenic patterns. Since the Fourier transform converts an arbitrary function to a summation of a series of sine and cosine terms of increasing frequency, the periodicity in an image can be detected as peaks in the frequency domain. The dominant frequency component should be equivalent to the inverse of the average interval.

In short, we computed the two-dimensional power spectrum of frequency by averaging power spectrums of several images, and calculated the distance between a peak and the origin. The actual procedure of this method was as follows: (1) to calculate the power spectrum of Fourier transform of several images (Fig. 2A-B), (2) to average the power spectrum images (Fig. 2C), and (3) to plot the average amplitude of the power spectrum at a certain distance from the origin (Fig. 2D). The resulting image should contain one peak near the origin, and the average interval could be obtained by the following value:

$$\frac{\text{Width of the image}}{\text{Distance between peak and origin}}$$

Peak length method

The Fourier transform is not sensitive enough when sharp peaks are not detected in the power spectrum, which frequently occurs in actual experimental data because they are distorted from ideal periodic patterns. Therefore, we developed a method to automatically measure the average interval in actual

chondrogenic patterns. The basic idea of this approach is as follows: we can estimate the pixels which contain peaks in a given image by segregating chondrogenic and nonchondrogenic areas and skeletonizing them. If we calculate the number of total pixels included in the area divided by the number of pixels which contains peaks, the value should represent the average number of pixels surrounding peak pixels (Fig. 3A). If we hypothesize that the peak pixels form a continuous line of one-pixel thick, it can be interpreted as the average interval between two neighboring peaks.

If the pattern is stripe-like, this method properly estimates the average interval because of the reason described above, but in actual experinental data the pattern is sometimes mesh-like or consists of spots according to the culture condition, which can seriously bias the result obtained by the method. In spot-like patterns, the amount of peak pixels becomes very low, and in stripe-like patterns it becomes very high. We circumvent these problems by detecting both peaks and valleys in a pattern and calculating the following value:

$$\frac{\text{Number of total pixels x 2}}{\text{Number of peak and valley pixels}}$$

If the chondrogenic pattern is spot-like, the pattern of undifferentiated area (valleys) becomes mesh-like, and vice versa. So the average of the peak and valley pixels in these two patterns is balanced and becomes close to the actual average interval.

The general procedure of this method was as follows (Fig. 3B): (1) to binarize an image to segregate chondrogenic and nonchondrogenic areas, (2) to skeletonize both chondrogenic and nonchondrogenic areas to identify the center line of each area, and (3) to calculate the number of total pixel / the number of peak and valley pixels x 2 to obtain the average interval between chondrogenic areas.

This method should satisfy the following requirements.

(1) The average interval calculated by this method should be closely correlated with the value obtained by manual measurement.

(2) The interval calculated by this method should depend solely on the distance between chondrogenic areas and must be stable over the following two types of variations that are frequently seen in actual chondrogenic patterns.

(a) The average interval calculated by this method must be independent of the pattern changes as shown in Fig. 5. Sometimes all the chondrogenic areas become continuous to form a mesh-like pattern, but they may be less continuous and form stripes or remain isolated from each other as islands. The average distance obtained by manual measurement was stable in any of these patterns produced by computer simulations (data not shown). The average interval calculated by the peak length method must reflect the corresponding value obtained manually or by Fourier transform in various patterns.

(b) The average interval calculated by this method must be independent of such factors as staining conditions and the degree of cell differentiation, which are not

related to the periodicity of the original pattern. In the peak length method, the effects of these factors are represented by the change in the threshold value to segregate chondrogenic and nonchondrogenic areas.

Simulations

To examine the feasibility of the method described above, computer simulation of the reaction-diffusion model was undertaken to generate idealized stripe or spot patterns. The reaction-diffusion model was originally proposed by Turing (1952), and the simulation used by Kondo and Asai (1995) was employed in this study. In short, this model hypothesizes the existence of two molecules, an activator and an inhibitor, and their activities are specified as follows: the activator promotes the production of both itself and the inhibitor, and the inhibitor suppresses the activator production and diffuses faster than the activator. As a result, the original small perturbation is amplified by the positive feedback of the activator, and the inhibitor laterally inhibits the activator's peak, which results in a stable periodic pattern. The simulation formulation and constants have been described by Kondo and Asai (1995).

Results

Correlation of the values obtained by the peak length method with those obtained by manual measurement and Fourier transform

To examine whether the peak length method described above can estimate the values obtained by well- established methods (manual measurement and Fourier transform), we analyzed the idealized simulation images by each method and compared the obtained values. The values obtained by the peak length method were closely correlated with the values obtained by manual measurement and Fourier transform. Fig. 4 shows the correlation between the average intervals obtained by the peak length method and the corresponding values obtained by manual measurement (A) and Fourier transform (B). Close correlations existed between the values obtained by the peak length method and those obtained by manual measurement and Fourier transform. Images that have various average intervals were generated by changing the diffusion coefficient in the reaction-diffusion model, and the average interval was calculated by manual measurement, the peak length method and Fourier transform. Fig. 4A demonstrates the relationship between the data obtained by manual measurement and by the peak length method. It can be noted that the obtained values are similar and highly correlated ($r=0.99$). Fig. 4B shows the correlation between the values obtained by the peak length method and by Fourier transform. The data obtained by the peak length method was highly correlated with those obtained by Fourier transform ($r=0.96$).

Stability of the peak length method

Under different experimental conditions, some variations were observed in chondrogenic patterns which were independent of the periodicity of the pattern.

To examine whether the values obtained by the peak length method are stable even in the existence of such variations, we artificially created two kinds of variations using a reaction-diffusion model and tested the stability of the values obtained by the peak length method. First, various patterns with the same average interval were created by changing a reaction parameter in the reaction-diffusion model. The patterns were sequentially changed from islands to stripes and further to mesh (Fig. 5) as we changed the parameter (Kondo and Asai, 1995). The average interval obtained by manual measurement and Fourier transform are identical in any of those patterns (data not shown), indicating that the average interval is not changed by this kind of pattern variation. Then the average intervals in these images were measured by the peak length method and it was shown that the average interval obtained by the peak length method did not change significantly (Fig. 5), indicating that the value obtained by the peak length method is not affected by this kind of pattern variation.

Next, the effect of thresholding on the peak length method was examined (Fig. 6). Several simulation patterns with the same average interval were created by the reaction-diffusion model, and they were binarized at various thresholds. The average interval of those images obtained by manual measurement and Fourier transform were identical in any of those threshold values (data not shown), indicating that the average interval is not changed by this kind of pattern variation. Then the average interval in each image was calculated using the peak length method (Fig. 6). The average interval did not show statistically significant changes, indicating that the average interval obtained by the peak length method is independent of the threshold values if the threshold value properly segregated the chondrogenic and nonchondrogenic areas.

Application of the proposed method to actual experimental data: comparison of reaction-diffusion and cell sorting models

To assess whether the peak length method can be applied to actual experimental data, we analyzed some experimental data by this method. There are two major models to explain the periodicity of the chondrogenic patterns in limb micromass culture. One is the reaction-diffusion model (Newman, 1996), in which the diffusion coefficent of the molecules is assumed to determine the periodicity of the pattern, and the other is the cell sorting model (Mochizuki et al.,1996), where cell adhesiveness and cell motility are supposed to determine the periodicity of the final pattern. To experimentally assess the validity of these models, we changed the culture conditions described above and assayed the resulting chondrogenic patterns by the peak length method.

Fig. 7 shows the average interval between chondrogenic areas under different diffusion coefficients, cell adhesiveness and cell motility. In Fig. 7A the diffusion coefficient of the culture was reduced by culturing cells in agarose gel. In Fig. 7B the adhesiveness of the cells was reduced by adding 0.25 mM EDTA in the culture medium. In Fig. 7C and 7D the general motility of the cells were reduced by exposing them at lower temperature (25 °C) during the pattern formation period (18-24 Hrs) or adding RGD peptide in the culture medium. In these cultures, a statistically significant decrease in the cell motility was observed by time-lapse recording ($p < 0.01$). It was shown that the change in the diffusion coefficient markedly decreased the average interval between chondrogenic areas, but in other cases the average interval was not changed significantly. The

difference of the average interval in Fig. 7A was statistically significant ($p<0.01$), but not in Fig. 7B-D.

Discussion

In the present study, we defined a simple parameter, the average interval between chondrogenic areas, which represents the periodicity in the chondrogenic patterns in micromass culture, and developed a practical method to automatically obtain the average interval between chondrogenic loci. The stability of the method was confirmed by comparing them with previously established methods, and an example of the application of this method was presented to compare validity of the two theoretical models of pattern formation.

The present results suggest that the analysis of the average interval can be used as a tool to elucidate the mechanisms of pattern formation during limb chondrogenesis. For example, we showed that the change in the diffusion coefficient altered the average interval while the change in cell adhesiveness and cell motility exerted no influence on the average interval, indicating that the reaction-diffusion model is more plausible as the pattern formation mechanism in this culture system. We could not completely rule out the cell sorting model because agarose gel also physically reduces the cell motility as shown in our previous paper (Miura and Shiota ,1999), and Fig. 7B-D shows a negative data because some other experimental condition which inhibits cell movement or cell adhesion more strongly may influence the pattern. Additional experiments are needed to determine the plausibility of the models, and the method described above should be a useful tool to analyze the resulting patterns.

An analysis of the effects of teratogenic agents on pattern formation in vitro should be useful to elucidate the pathogenetic mechanisms of limb malformations, and the method we developed which automatically measures the average interval should be a useful tool to routinely analyze the pattern changes in vitro. For example, ethanol has been shown to induce polydactyly in offspring when it is given to pregnant animals (Pauli et al., 1986; West et al. 1981). Kulyk and Hoffman (1996) showed that ethanol exposure markedly promotes the differentiation of chondrocytes in vitro. In their figures it was clearly seen that the average interval between stripes was significantly reduced by ethanol exposure, which was not properly explained in their paper. Although Pauli et al. (1986) claimed that polydactyly in vivo may be formed by ethanol-induced impairment of vascular supply, it is difficult to explain why *more* chondrogenic sites are formed by anoxia. If the pattern change observed in vitro has something to do with the pathogenesis of polydactyly, we could explain both of their observation as follows: The mechanism to establish the periodicity of the chondrogenic pattern is impaired by ethanol, and the intervals between chondrogenic areas is decreased in a limb bud of the same size, which eventually produces more chondrogenic loci. Moreover, by examining the effects of various agents on pattern formation in vitro, it may be possible to idenitify chemicals that can possibly affect limb morphogenesis.

This method can also be applied to other experimental systems which show periodic pattern formation as skin organ culture (Jung et al., 1998), although careful experimental setting is needed to eliminate the effect of artifacts. For example, if the cultured tissue grows outward unlimitedly, it should increase the

average interval of a predetermined pattern, which can be misunderstood as an effect on periodic pattern formation. We circumvented such a problem in our culture system by making a circular incision on the collagen-coated dish and culturing cells inside the circle. Soon after the onset of the culture, cells became confluent inside the circle and did not grow beyond the circular insicion because it acted as a physical barrer or the extracellular matrices were lacking on the incision line.

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Appendix: Macro Program to measure the average interval

```
VAR
i,j:Integer;
x,y,w,h:integer;
Stack,original:Integer;
scale, aspectRatio:real;
unit:string;



Procedure Preprocess;
begin

Smooth;smooth;smooth;smooth;smooth;smooth;
end;


Macro 'Calculate average interval[a]'

Begin

original := PidNumber;

GetRoi(x,y,w,h);
SetNewSize(w,h);
MakeNewStack('Stack');
stack := PidNumber;
SelectPic(original);

getScale(scale, unit, aspectRatio);
setUser1Label(unit);

For i :=1 to nSlices Do begin
SelectSlice(i);
Duplicate('untitled');
Preprocess;
EnhanceContrast;
ApplyLUT;
AutoThreshold;
MakeBinary;
skeletonize;
ResetCounter;
Measure;
rUser1[i] := rMean[1];

SelectAll;
Copy;
Dispose;
SelectPic(Stack);
Paste;
SelectPic(Original);

Duplicate('untitled');
Invert;
Preprocess;
EnhanceContrast;
ApplyLUT;
```

```
AutoThreshold;
MakeBinary;
skeletonize;
ResetCounter;
Measure;

rUser1[i] := rUser1[i] + rMean[1];

rUser1[i] := 255 -(rUser1[i] * scale) * 2;

SelectAll;
Copy;
Dispose;
SelectPic(Stack);
Paste;
DoOr;


SelectPic(Original);
Copy;
SelectPic(Stack);
Paste;
DoOr;
AddSlice;
SelectPic(Original);
end;


SelectPic(stack);
DeleteSlice;

SetCounter( nSlices);
SetOptions('user1');
ShowResults;
end;
```

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Legends

Figure 1
A chondrogenic pattern formed in micromass culture of limb bud cells and the definition of the average interval. (A) A chondrogenic pattern in limb micromass culture. Limb bud cells of day 10.5-mouse embryos were dissociated and cultured for 5 days. Chondrocytes on the dish were stained with Alcian blue. (B) The average interval was defined as an average of intervals between the center lines of neighboring chondrogenic areas. Arrows indicate measured intervals. Note that the interval does not depend on the width of chondrogenic areas.

Figure 2
Explanation of the Fourier transform. (A) Original images. (B) The power spectrum of each image was calculated. (C) The average of these power spectrums was calculated. Usually, a circular peak was observed around the origin. (D) A profile plot of the power spectrum was created and the distance between the two peaks was measured. The average interval can be calculated by dividing the width of the image by the distance between the peak and the origin in the profile plot.

Figure 3
Qualitative explanation of the peak length method. (A) The total pixels per peak pixels in a given area represents the average area which surrounds one peak pixel. If we hypothesize that the peak pixels form a one-pixel thick continuous line, it can be interpreted as the average interval between two neighboring peaks. (B) Detection of the peaks and valleys in chondrogenic patterns. First, images were binarized to segregate chondrogenic and nonchondrogenic areas. Then binarized images and inverted images of the binarized images were skeletonized to detect both peaks and valleys (black Pixels) , and the total number of black pixels was calculated.

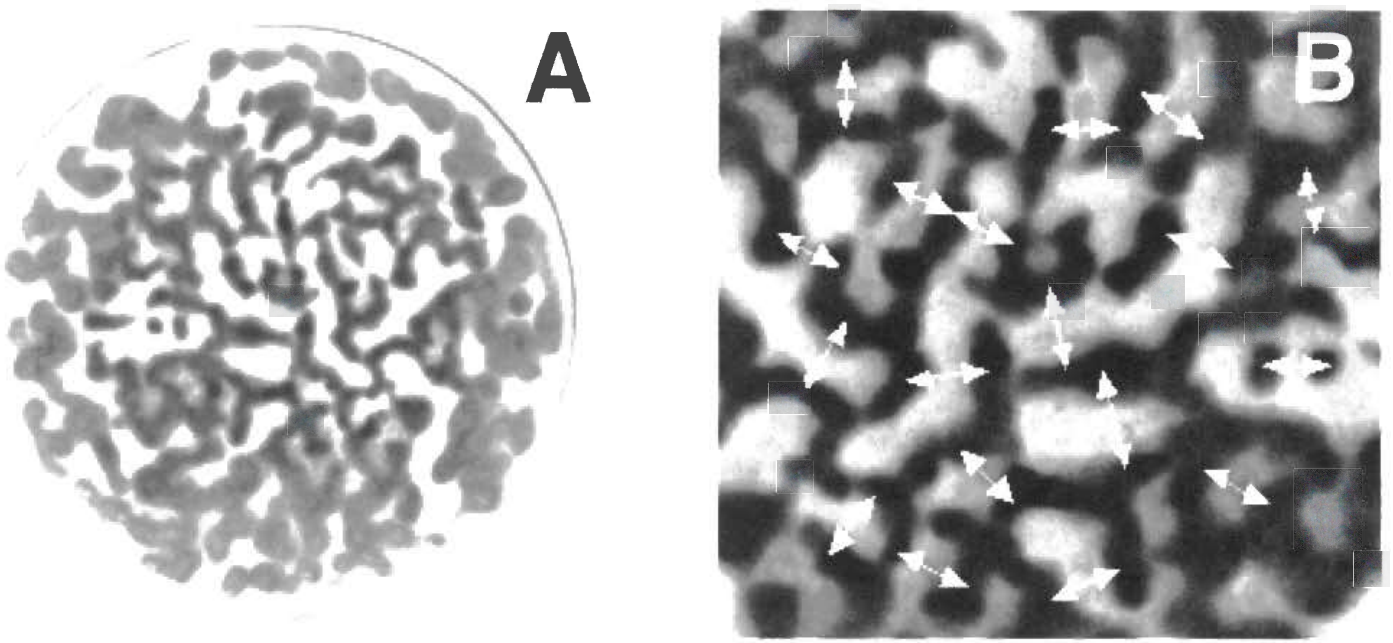
Figure 4
Relationship between the values obtained by the peak length method and those obtained by manual measurement and Fourier transform. (A) Correlation between the values obtained by manual measurement and by the peak length method. A close correlation was observed between the two values. Correlation coefficient = 0.99. (B) Correlation between the values obtained by the peak length method and by the Fourier transform. A close correlation was observed between the two values. Correlation coefficient = 0.96.

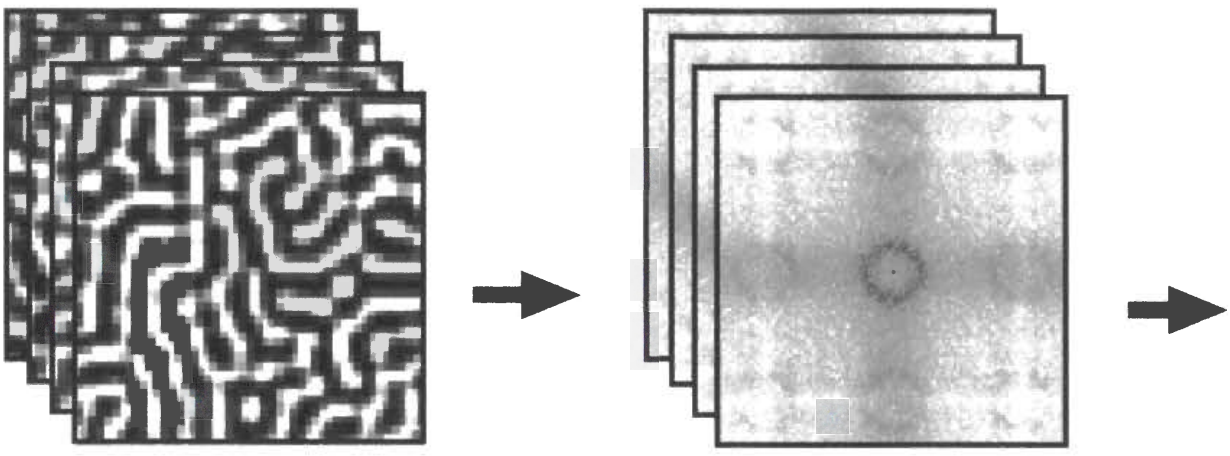
Figure 5
The average intervals obtained by the peak length method are nearly identical to those obtained by manual measurement or Fourier transform over a wide range of pattern changes. The average interval was calculated for pattern variations which are frequently observed in actual experiments. The results were compared with the value obtained by manual measurement, and the ratio of the two values was around 1.0. No statistically significant difference was noted among these patterns.

Fugure 6
The average interval obtained by the peak length method is stable over a wide range of threshold values. The threshold value represents the degree of cell differentiation or the intensity of Alcian blue staining. The peak length of an image was calculated by using various threshold values, but the average interval was not changed significantly provided the values properly segregated chondrogenic and nonchondrogenic areas. No statistically significant difference was detected among these patterns.

Figure 7
Examples of application of the peak length to actual experimental data. (A) A decreasing diffusion coefficient caused a change in the average interval. In the treated group the cells were overlaid with 0.5% agarose gel, while the control culture was performed in a liquid medium. A statistically significant difference was detected between the average intervals by the Mann-Whitney method ($P<0.01$). (B) Changing cell adhesiveness did not induce a change in the average interval. In the treated culture 0.25 mM EDTA was supplemented in culture medium, which should inhibit calcium-dependent cell adhesion. No statistically significant difference of the average interval was observed. (C, D) Changing cell motility did not influence the average interval. In (C), RGD peptide was supplemented to the culture medium, which should inhibit integrin-mediated adhesion

and cell movement. In (D), culture was carried out at 25 °C during 18-24 Hrs of culture. In both cases, although the cell motility is significantly decreased, the average interval was not altered significantly.


A
B



A

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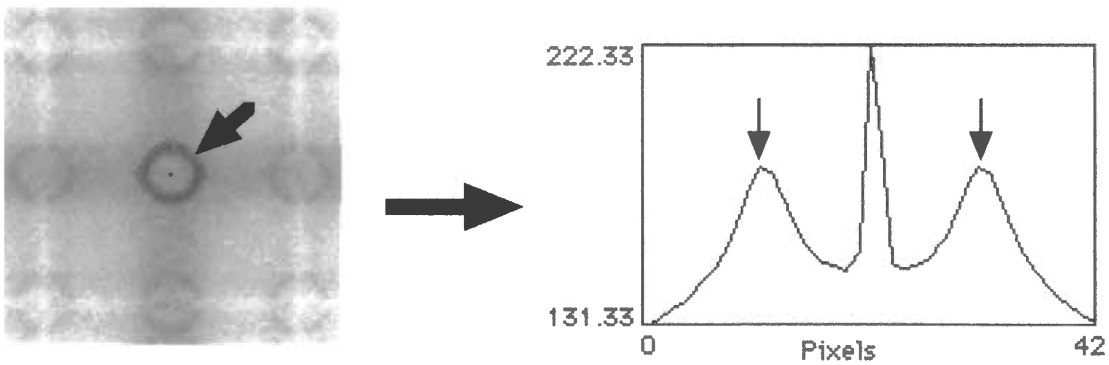
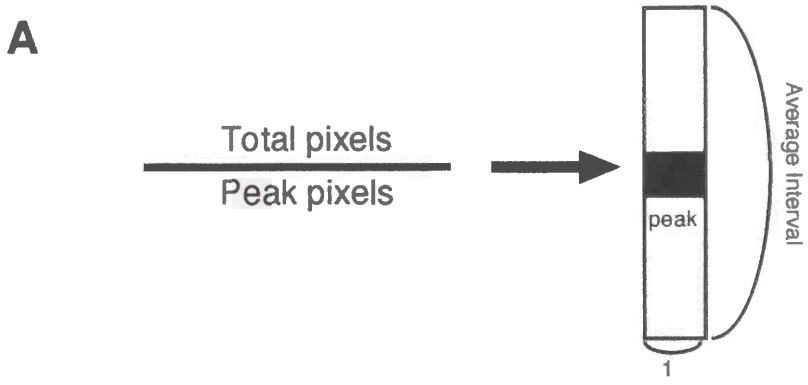
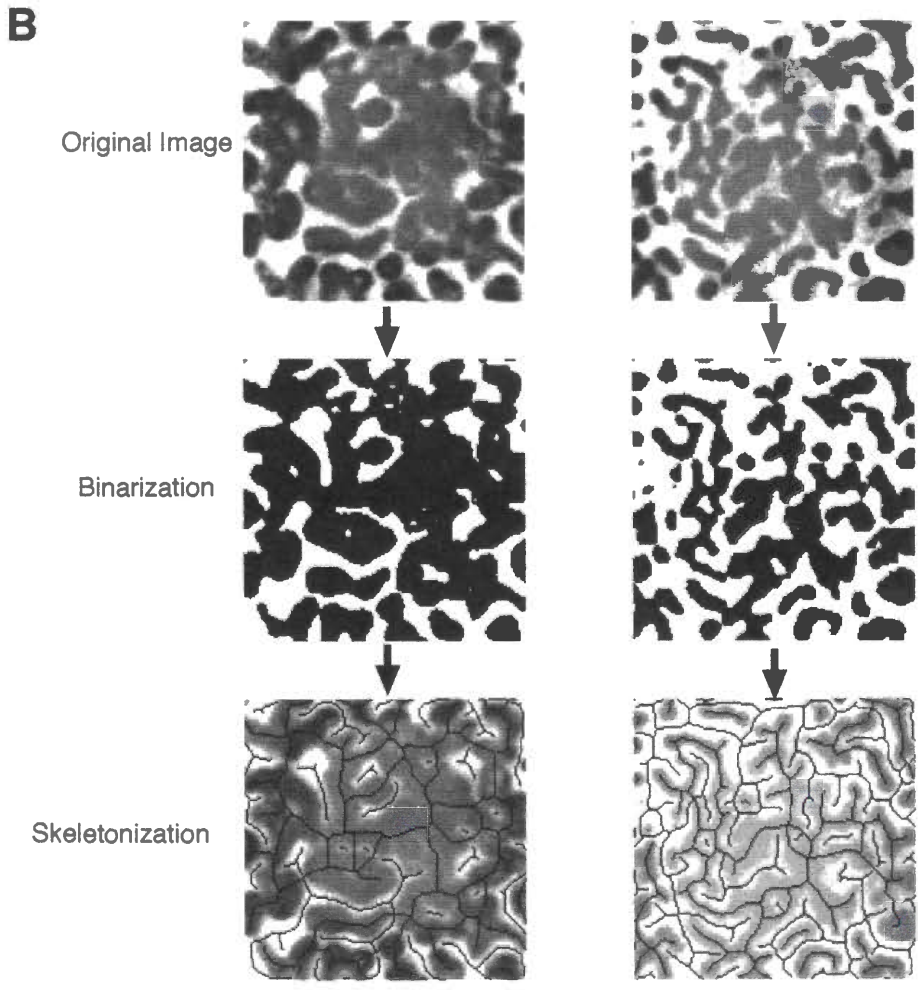


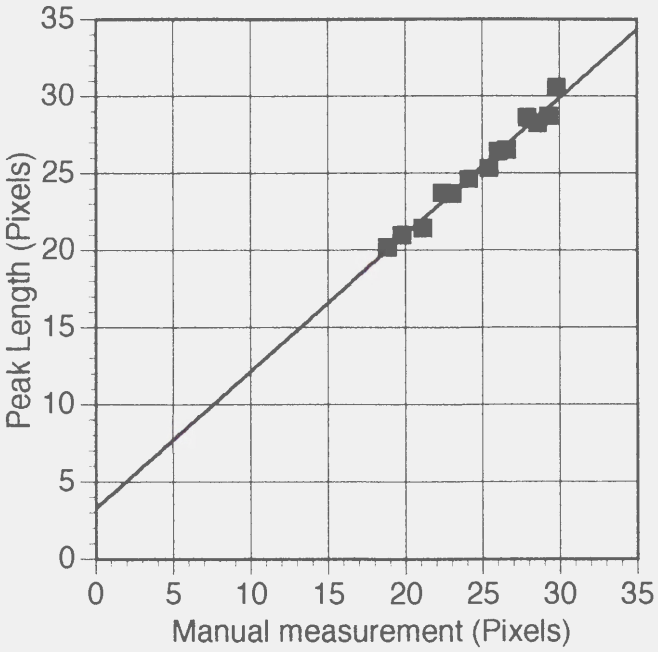
C

D


A
Total pixels
Peak pixels
peak
Average Interval
1

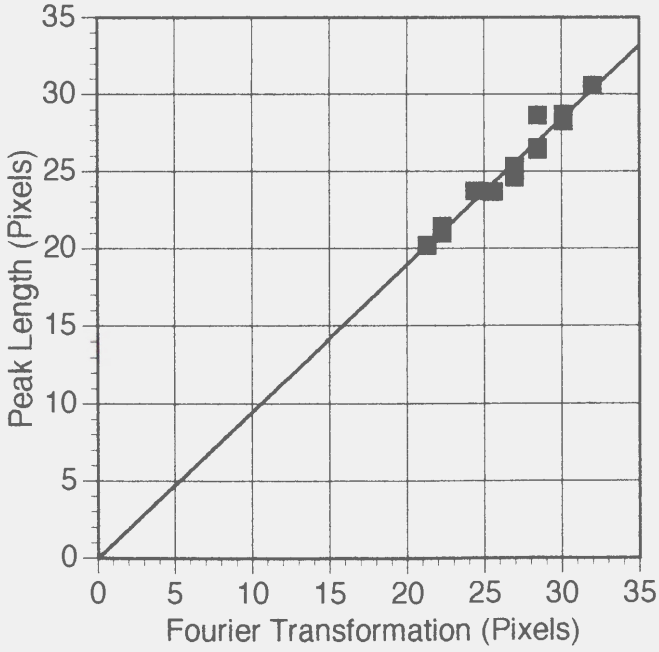

B
Original Image
Binarization
Skeletonization

A

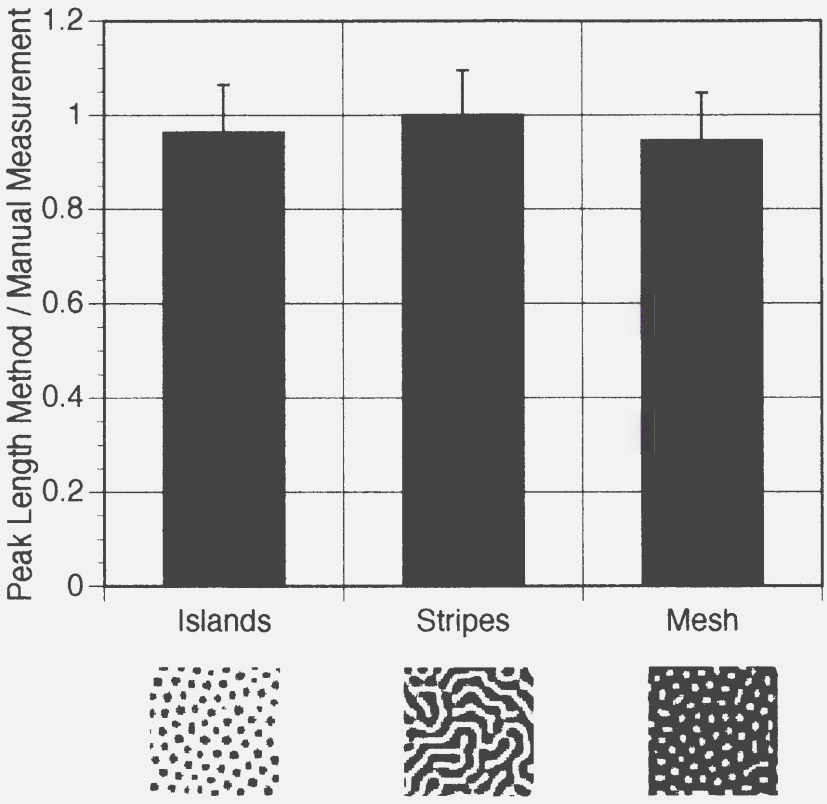

Peak Length (Pixels)
Manual measurement (Pixels)
0
5
10
15
20
25
30
35

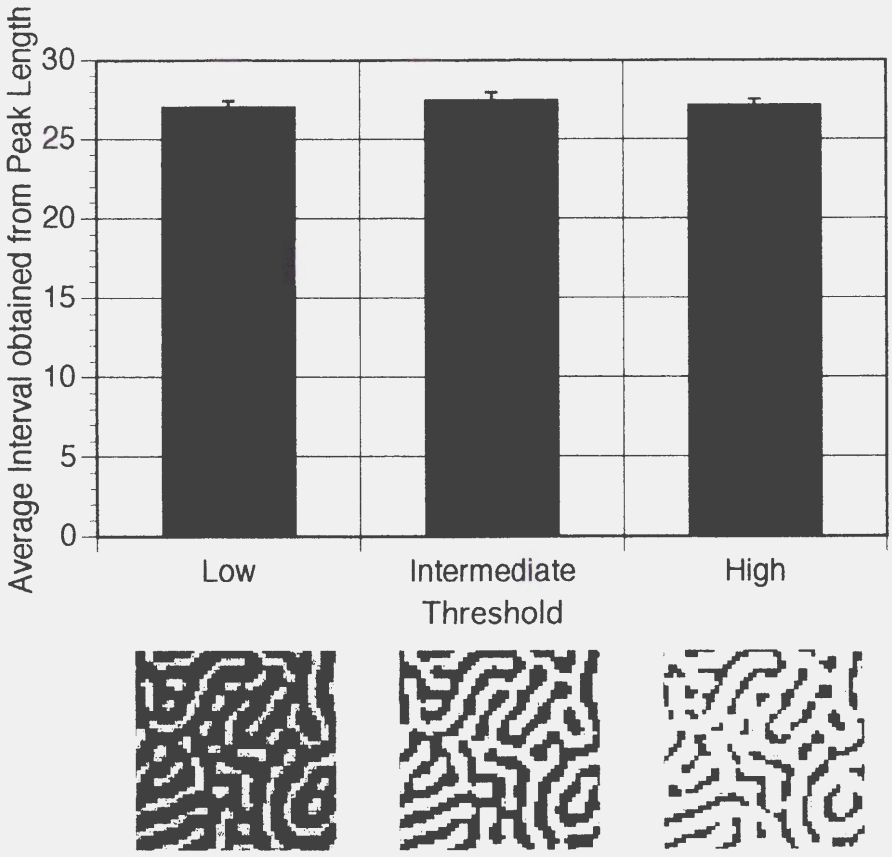
r = 0.99

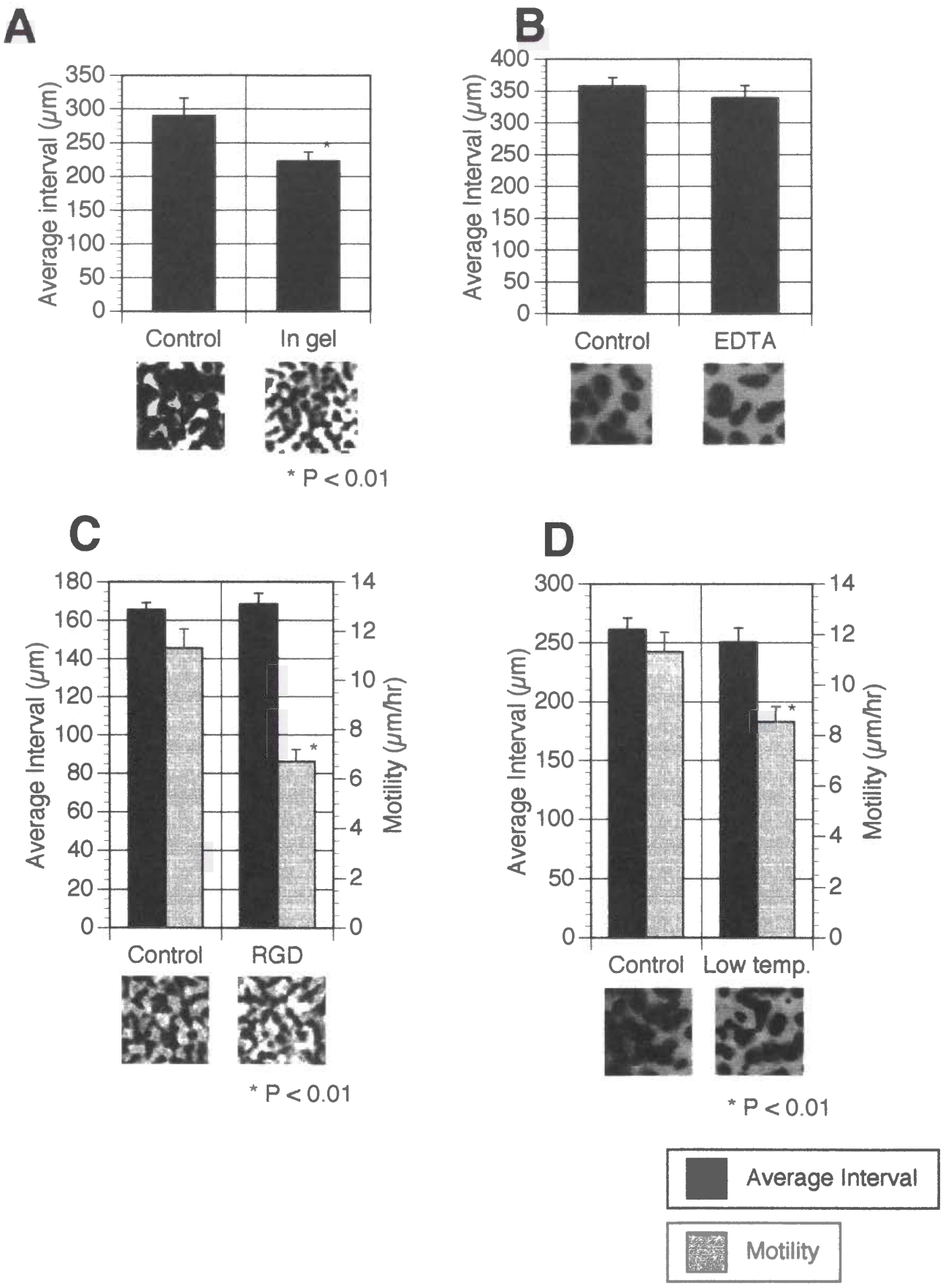
B


Peak Length (Pixels)
Fourier Transformation (Pixels)
0
5
10
15
20
25
30
35

r = 0.96


Peak Length Method / Manual Measurement
1.2
1
0.8
0.6
0.4
0.2
0
Islands
Stripes
Mesh


Average Interval obtained from Peak Length
30
25
20
15
10
5
0
Low
Intermediate
High
Threshold


A
Average interval (μm)
350
300
250
200
150
100
50
0
Control
In gel
*
* P < 0.01
B
Average Interval (μm)
400
350
300
250
200
150
100
50
0
Control
EDTA
C
Average Interval (μm)
180
160
140
120
100
80
60
40
20
0
Motility (μm/hr)
14
12
10
8
6
4
2
0
Control
RGD
* P < 0.01
D
Average Interval (μm)
300
250
200
150
100
50
0
Motility (μm/hr)
14
12
10
8
6
4
2
0
Control
Low temp.
* P < 0.01
Average Interval
Motility